

***eRF3a/GSPT1* 12-GGC allele increases the susceptibility for breast cancer development**

JOANA MALTA-VACAS¹, CELINE CHAUVIN⁵, LUCILIA GONÇALVES²,
ANTONIA NAZARÉ³, CARLOS CARVALHO³, CAROLINO MONTEIRO⁴,
DENYSE BAGREL⁶, OLIVIER JEAN-JEAN⁷ and MIGUEL BRITO¹

¹Escola Superior de Tecnologia da Saúde de Lisboa, 1990-096 Lisboa; Departments of ²Pathology and

³Oncology, Hospital Fernando Fonseca, 2700 Amadora; ⁴Faculdade de Farmacia, Universidade de Lisboa, 1649-003 Lisbon, Portugal; ⁵INSERM U845, Faculté de Médecine Necker-Enfants Malades, Université Paris 5, 75015 Paris;

⁶Laboratoire d'Ingénierie Moléculaire et Biochimie Pharmacologique, UFR SciFA, Université de Metz, Metz;

⁷UPMC Univ Paris 06, UMR 7098, CNRS, F-75005 Paris, France

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Abstract. It is now widely recognized that translation factors are involved in cancer development and that components of the translation machinery that are deregulated in cancer cells may become targets for cancer therapy. The eukaryotic Release Factor 3 (eRF3) is a GTPase that associates with eRF1 in a complex that mediates translation termination. *eRF3a/GSPT1* first exon contains a (GGC)_n expansion coding for proteins with different N-terminal extremities. Herein we show that the longer allele (12-GGC) is present in 5.1% (7/137) of the breast cancer patients analysed and is absent in the control population (0/135), corresponding to an increased risk for cancer development, as revealed by Odds Ratio analysis. mRNA quantification suggests that patients with the 12-GGC allele overexpress *eRF3a/GSPT1* in tumor tissues relative to the normal adjacent tissues. However, using an *in vivo* assay for translation termination in HEK293 cells, we do not detect any difference in the activity of the eRF3a proteins encoded by the various *eRF3a/GSPT1* alleles. Although the connection between the presence of *eRF3a/GSPT1* 12-GGC allele and tumorigenesis is still unknown, our data suggest that the presence of the 12-GGC allele provides a potential novel risk marker for various types of cancer.

Introduction

There is presently a growing body of evidence that supports the involvement of translation in cell proliferation and cancer development (1-3). This includes overexpression of initiation (4), elongation (5), and more recently, termination factors (6).

Eukaryotic translation termination is governed by two release factors, eRF1 and eRF3, that associate in a complex which binds to the ribosomal A site. eRF1 recognises the three stop codons, and promotes the release of the nascent polypeptide chain. eRF3 is a small GTPase that enhances eRF1 activity (7). The C-terminal region of eRF3 proteins is highly conserved and essential for translation termination and interaction with eRF1 whereas the N-terminal region varies in both length and sequence among species. Although this domain has been reported to be dispensable for translation termination (7,8), it was recently shown that it influences eRF3 functions in translation and possibly in other cellular processes (9,10).

In humans, eRF3 has two distinct isoforms, eRF3a encoded by *eRF3a/GSPT1* gene located in 16p13.1 (11) and eRF3b, encoded by *eRF3b/GSPT2* gene located in Xp11.21-23 (12). eRF3a mRNA is abundant in all tissues and its level varies during the cell cycle, whereas eRF3b mRNA is poorly expressed in most mouse tissues tested, except in brain (13). It was previously reported that *eRF3a/GSPT1* mRNA level is increased in 70% of the intestinal type gastric tumors (6) and strongly decreased during human chondrocyte differentiation (14).

eRF3a and eRF3b proteins share 87% identity, most of the differences being concentrated in their N-terminal domains. The N-terminal domain of human eRF3a contains a polyglycine expansion encoded by a stable (GGC)_n expansion in *eRF3a/GSPT1* first exon. There are five known alleles, which encode 7, 9, 10, 11 and 12 glycines. Our group has reported a strong association between the longest allele (12-GGC) and gastric cancer development (15), which was shown to be a germline mutation. Because of the absence

Correspondence to: Professor Miguel Brito, Escola Superior de Tecnologia da Saúde de Lisboa, Av. D. João II lote 4.69.01, 1990-096 Lisbon, Portugal
E-mail: miguel.brito@estesl.ipl.pt

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of the 12-GGC allele in healthy control population, it was suggested that the presence of *eRF3a/GSPT1* 12-GGC allele was directly associated with gastric cancer development.

In the present study, we aimed to evaluate *eRF3a/GSPT1* as a susceptibility locus for cancer development, searching for further evidence in other cancer models. Breast cancer was chosen because it is one of the most common and serious malignancies affecting women. In addition, more than 75% of the hereditary tumors result from inherited defects in genes that are still to be identified (16).

Breast cancer patients were genotyped for the (GGC)_n polymorphism in order to determine the frequency of the 12-GGC allele and the associated cancer risk. We also investigated the correlations between the different GGC alleles and the pattern of *eRF3a/GSPT1* expression in tumor samples. In addition, we looked for correlations between clinical/morphological parameters, the *eRF3a/GSPT1* genotype and *eRF3a/GSPT1* gene expression levels. Finally, a functional *in vivo* assay was used to search for differences in the translation termination activity of eRF3a proteins encoded by the different GGC alleles.

Material and methods

Patients and sample collection. We analysed 137 breast tumors collected from surgical resections in Fernando da Fonseca Hospital, after informed consent. This study was approved by the Scientific Committee of the hospital. The tumors were diagnosed as breast cancers by histopathological examination performed in the department of Surgical Pathology. A sample of 135 healthy blood donors with matched demographic characteristics was used as control population.

Freshly frozen samples from 52 tumors, together with a sample from the adjacent non-neoplastic tissue, were collected for gene expression analysis. Those tissue samples were stored in RNAlater (Ambion, Austin, TX, USA) at -20°C immediately after surgery until RNA extraction. Data were obtained regarding grade of differentiation, tumor size, node status, vascular invasion, surgical margins and previous chemo/radiotherapy treatment.

Immunohistochemistry. Paraffin-embedded sections (thickness, 2 µm) of formalin-fixed paraffin-embedded tissue samples were used for immunocytochemistry. A streptavidin-biotin immunoperoxidase staining technique was performed using the Dako Autostainer Plus® and specific antibodies for c-erb-B2 (Dako®), Estrogen Receptor (EgR NeoMarkers®) and Progesterone Receptor – A/B forms (PgR Novocastra®).

A polyclonal anti-human c-erbB-2 oncoprotein and a monoclonal rabbit (Eg) and a monoclonal mouse (Pg) antibody were used in this study. The antibody c-erbB-2 labels an intracellular domain of this oncoprotein and a specific stain is confined to the cell membrane. For estrogen and progesterone label a nuclear staining was considered specific. A pre-treatment of tissue sections with heat-induced epitope retrieval is required. For the staining procedure we used EDTA buffer, pH 8.0, keeping the tissue sections in a moist atmosphere. Antibody at a dilution of 1:500 (c-erb-B2), 1:100

(Eg) and 1:300 (Pg) in DakoCytomation Antibody Diluent was used with 25 min heat-induced (98°C) epitope retrieval in EDTA, pH 8.0, and 30-min incubation at room temperature with the primary antibody. Visualization was done using Dako EnVision™ +/HRP kit code no. K 4010 (c-erb-B2 and Eg) and Dako EnVision™ +/HRP kit code no. K 4006 (PgR).

DNA extraction, RNA extraction, and cDNA synthesis. DNA was extracted either from two 7 mm thick formalin fixed, paraffin wax embedded tissue sections using chelex resins (17) or from total blood using the DNeasy tissue kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol.

RNA was extracted from fresh tissues using the SV Total RNA Isolation System (Promega, Mannheim, Germany), as recommended by the manufacturer. First strand cDNA was synthesised using MultiScribe™ reverse transcriptase (Applied BioSystems, Foster City, CA, USA), with random hexamers, according to the manufacturer's instructions, in a total volume of 50 µl; samples were incubated for 10 min at 25°C, 30 min at 48°C, and finally for 5 min at 95°C.

Patients genotyping. A fragment of *eRF3a/GSPT1* exon 1 including the (GGC)_n polymorphism was PCR amplified, from patient's genomic DNA, using a 6'-FAM labelled forward primer and a non-labelled reverse primer as previously described (15). Fragment sizes were detected in an ABI PRISM 3700 DNA Analyzer sequencer and analysed with GeneScan 3.7. software (ABi).

Relative gene expression. Expression of the eukaryotic translation factors eRF3a, eRF3b, and eRF1 in tumor samples relative to normal adjacent tissues was investigated using real-time quantitative RT-PCR using gene-specific primers and TaqMan probes. All the samples were analysed in triplicate as described by Malta-Vacas *et al.* (5).

Relative quantification of the mRNA levels of the target genes (quantity of transcripts of the target in tumor samples relative to normal tissues) was determined using the $\Delta\Delta CT$ method (18). Briefly, the amount of target was normalised to the endogenous reference gene (18S rRNA) and its expression in tumor samples was calculated relative to a calibrator (normal adjacent sample). Final results are expressed as N-fold difference in tumor expression relative to non-neoplastic adjacent tissue.

Plasmid constructions and cell line

Plasmids. Plasmid expressing small interfering RNA si-3a1 targeting eRF3a mRNA and plasmid pCMV-heRF3a expressing human eRF3a have been previously described (19).

The plasmid pCMV-heRF3a was used to construct the plasmids expressing the different GGC allele of eRF3a. *eRF3a/GSPT1* exon1 fragments with GGC repeat length of 7, 9, 10, 11, and 12 were amplified by PCR from genomic DNA of genotyped patients, using 5'-CCATTTCTCGCTCTCTGTCCACC-3' and 5'-ACTCAACGTCAACGCCAAG-3' primers. The PCR fragments were digested with *Xma*I and *Bam*HI, purified, and ligated into the *Xma*I/*Bam*HI fragment of pCMV-heRF3a. The five eRF3a expression plasmids

obtained were sequenced to verify the correct sequence and GGC repeat length of the inserts.

Cell culture and electroporation. The 559C cell line is a derivative of human HEK293 cell line stably expressing a *lacZ* gene in which the coding sequence is interrupted by a TAG stop codon. Cell cultures and electroporation was performed as previously described (19). Briefly, the 559C cells were depleted in eRF3a by electroporation of a plasmid expressing a siRNA (si-3a1) directed against eRF3a mRNA or pSuper as a control and re-electroporated 3 days later with either the empty vector pBK-CMV (3a1/pBK and sup/pBK) or plasmid expressing the eRF3a variants (3a1/7GGC, 3a1/9GGC, 3a1/10GGC, 3a1/11GGC, and 3a1/12GGC).

The antibodies against human eRF3a and eRF1 were previously described (19). The anti- α -tubulin (DM1A), and anti-rabbit IgG and anti-mouse IgG peroxidase-linked antibodies were from Amersham Biosciences. Western blotting were performed as previously described (19).

Readthrough assay. The pellets of electroporated cells were lysed by freeze-thawing cycles and cell lysates were assayed for total proteins and β -galactosidase activity. For each sample, total protein concentration was determined using the Micro BCA Protein Assay Reagent Kit (Pierce). The β -galactosidase assays were performed using the Luminescent betagalactosidase Detection Kit II (Clontech), following the manufacturer's instructions. The β -galactosidase activity was measured as relative light units (RLU) using a single photon count program on scintillation counter. The β -galactosidase activity was expressed as RLU/mg of total proteins and the readthrough efficiencies were calculated as sample activity relative to negative control activity (cell extract 3a1/pBK). Thus, for each experiment, the percent readthrough level relative to readthrough level of cells electroporated with plasmid expressing si-3a1 and re-electroporated with pBK-CMV (3a1/pBK) was calculated and expressed as read-through efficiency.

Statistical analysis. Allele frequencies and Fisher's tests were calculated in GENETOP (version 1.2) (20). Odds ratios were calculated according to (21). All other statistical analysis were performed in SPSS 14.0 for Windows. Spearman's Rho coefficient was used to investigate correlations between the patient's genotypes and gene expression levels, and also between both genetic parameters and clinical parameters. In all cases, the tests were considered significant when the p-values were <0.05.

Results

eRF3a/GSPT1 GGCn alleles in breast cancer patients. The genotypes of 137 breast cancer patients and 135 healthy blood donors were determined by fluorescent PCR amplification and peak detection in an automatic sequencer. For each patient, normal and tumor tissue samples were analysed and no discrepancies were detected. Therefore, the allele length variations were considered germline mutations and validated for comparison with the frequencies obtained from blood samples analysis for the control group.

The five known *eRF3a/GSPT1* GGC alleles corresponding to 7, 9, 10, 11 and 12 GGC repeat units were identified. The 10-GGC allele was the most frequent in both groups (Fig. 1A). Note that repetitive sequencing revealed that the 8-GGC allele (15) carries only 7 GGC repeats and was renamed 7-GGC.

Using the Fisher's test, we detected significant differences between the breast cancer and control populations in genotypic frequencies ($\chi^2=6.618$; $p=0.03655$) but not in allelic frequencies ($\chi^2=5.535$; $p=0.06282$). The 12-GGC allele, which was only detected in cancer patients (allelic frequency=0.025), was associated with an 11-fold increase in breast cancer risk (OR=11.32; CI=0.64-199.93). Regarding genotypes, the 12-GGC allele was presented in two different genotypes, 10-12GGC and 11-12GGC (Fig. 1B), which were associated with 6.26- and 4.87-fold increased risk for breast cancer, respectively.

eRF3a/GSPT1 mRNA expression in tumor tissues. Relative expression of *eRF3a/GSPT1* gene was determined by real-time RT-PCR using gene specific primers and TaqMan probes in 52 breast cancer patients. *eRF3a/GSPT1* was classified as overexpressed when its mRNA level in tumor tissue samples was at least the double of that in adjacent non-neoplastic tissues. Underexpression was defined as less than half the expression level relative to adjacent non-neoplastic samples. The results showed that *eRF3a/GSPT1* was overexpressed in 44% (23/52) of the tumor samples whereas it was underexpressed in 20% (10/52) of the samples. Moreover, in 17% (9/52) of the tumors the expression level of *eRF3a/GSPT1* was >5-fold higher than in non-neoplastic adjacent tissue.

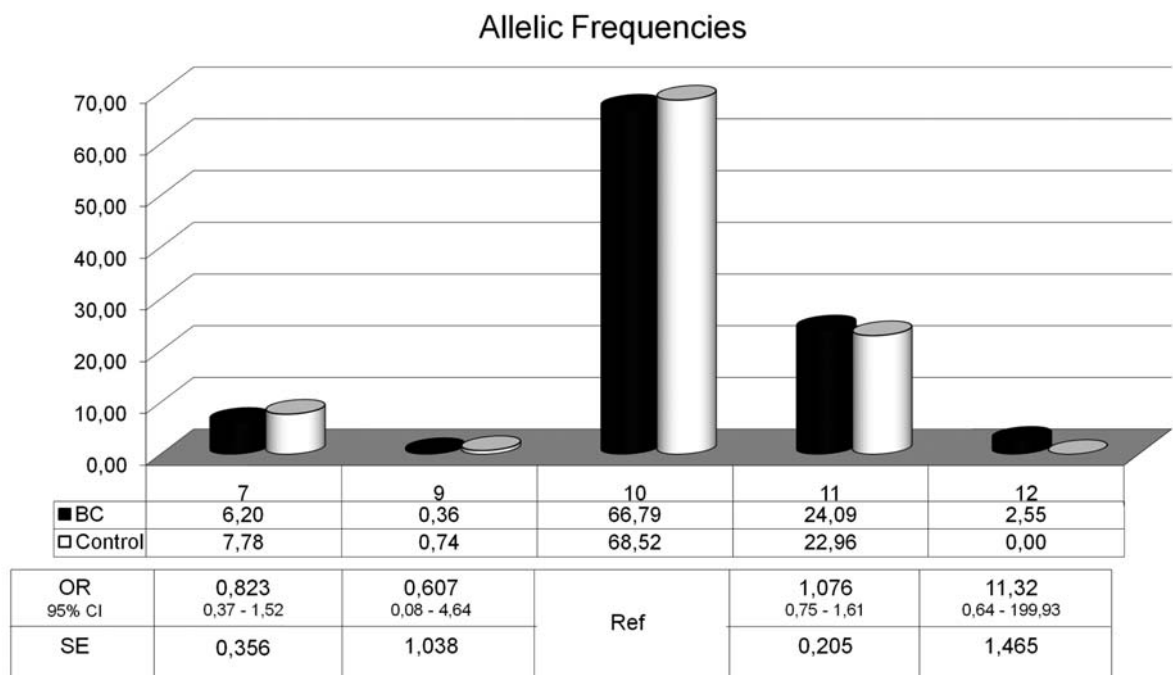
To explore if different *eRF3a/GSPT1* expression levels corresponded to different tumor types, a subset of 27 invasive ductal carcinomas (IDC) was analysed, but the same expression pattern was observed – 44% (12/27) of the tumors overexpressed *eRF3a/GSPT1* (Fig. 2A). This suggests that the variations of *eRF3a/GSPT1* mRNA expression are not specific for a particular type of tumor.

eRF3b/GSPT2 mRNA levels were also quantified to determine whether the changes in *eRF3a/GSPT1* levels were balanced by modifications in *eRF3b/GSPT2* gene expression. We observed that *eRF3b/GSPT2* mRNA was undetectable in normal tissue samples and only poorly expressed in 25% (7/27) of the tumor tissues. There was no correlation between *eRF3a/GSPT1* and *eRF3b/GSPT2* expression levels.

Because eRF1 is the partner of eRF3 in the translation termination complex, we also examine the expression status of eRF1 mRNA. eRF1 mRNA was overexpressed in 33% of the tumor samples, underexpressed in 22% and we did not detect any variation between normal and tumor tissues in 44% (12/27) of the cases analysed (Fig. 2B). However, no significant correlation was found between the variations of eRF1 and *eRF3a/GSPT1* mRNA levels (Fig. 2).

In addition, we examined a set of biomarkers that are currently used to determine the prognosis and treatment of breast cancer, i.e., c-erb-B2, ER and PR proteins expression (22), and the status of different clinical parameters, grade of differentiation, tumor size, node status, vascular invasion, surgical margins and previous chemo/radiotherapy treatment (data not shown). We did not detect any significant correlation

A



B

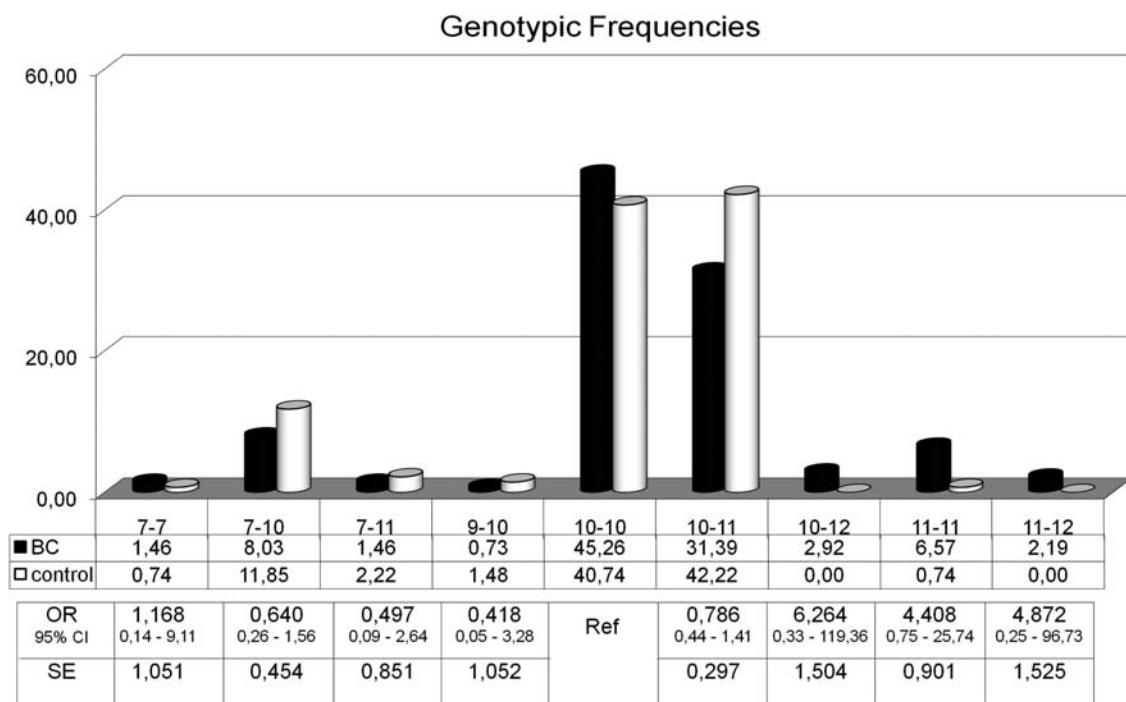


Figure 1. Frequencies of *eRF3a/GSPT1* polyglycine repeat length alleles and genotypes in 96 breast cancer patients and 135 control individuals. Fragment lengths were determined by fluorescent PCR amplification and peak detection in an automatic sequencer. (A) Allelic frequencies and Odds Ratio analysis for the *eRF3a/GSPT1* (GGC)*n* alleles; (B) Genotypic frequencies and Odds Ratio analysis for the *eRF3a/GSPT1* (GGC)*n* genotypes. BC, breast cancer; OR, odds ratio; CI, confidence interval; SE, standard error.

between these parameters and the variations of *eRF3a/GSPT1* mRNA levels.

Allele-specific expression of eRF3a/GSPT1 mRNA. In order to determine whether the GGC repeat length influence *eRF3a/GSPT1* expression or whether variations of *eRF3a/GSPT1* expression were linked to a specific GGC allele, we examined the genotype of the 52 patients for whom the expression status of *eRF3a/GSPT1* in tumor tissues has been determined. We failed to find significant correlations

between the GGC repeat length and the levels of *eRF3a/GSPT1* transcripts in breast cancer patients, when the 52 patients analysed were pooled (Table I). However, almost 60% of the patients with the 7-10GGC genotype revealed underexpression of *eRF3a/GSPT1*. Regarding the subset of the 27 patients with IDC type tumors, all 3 patients carrying the 7-GGC allele underexpressed *eRF3a/GSPT1* mRNA in tumor samples (samples identified with * in Fig. 2A). Moreover, these tumor tissues were positive for c-erb-B2 immunoexpression. At the opposite, the two 12-GGC alleles

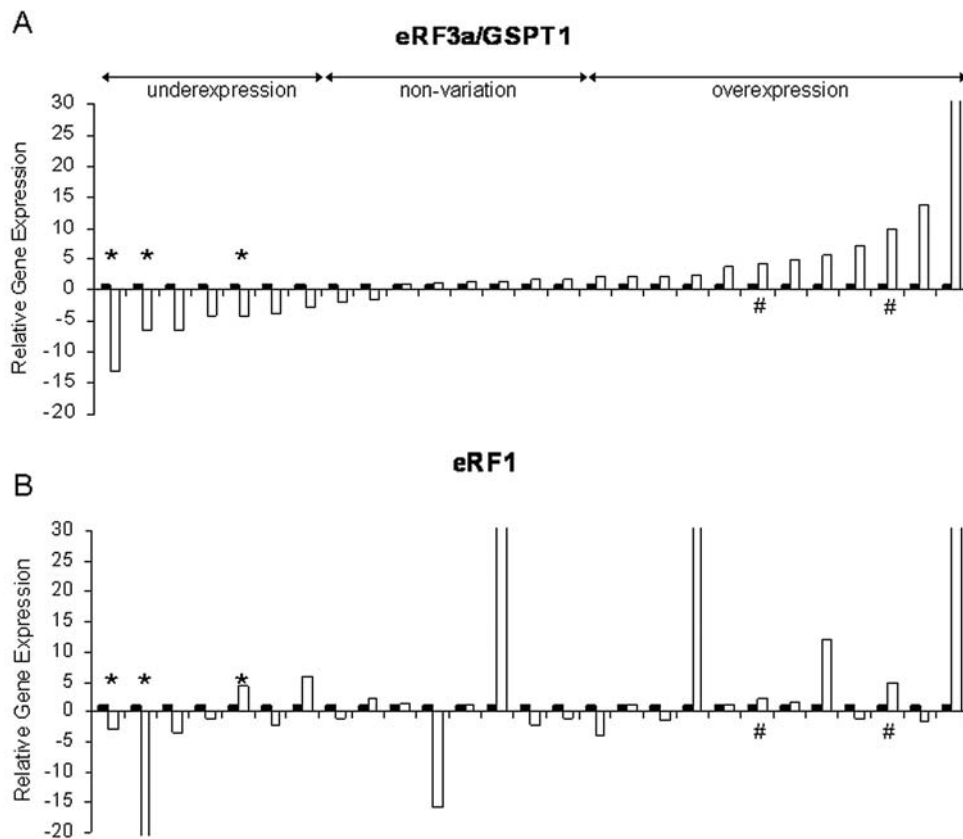


Figure 2. *eRF3a/GSPT1* and eRF1 relative gene expression levels in breast tumors. Relative expression of *eRF3a/GSPT1* (A) and eRF1 (B) at the mRNA level was quantified in 27 IDC breast tumors (open bars) relative to normal adjacent tissues (black bars). Relative quantification was determined by real-time RT-PCR using gene-specific primers and TaqMan probes, using the $\Delta\Delta C_T$ method. Results are expressed as N-fold expression in tumor relative to normal tissue. *Samples with the 7-GGC allele; #samples with the 12-GGC allele.

Table I. *eRF3a/GSPT1* gene expression levels in 52 breast cancer patients.

	N	Expression (%)		
		OE	NV	UE
Alleles (GGC)n				
7	10	30	30	40
9	0			
10	73	43.8	35.6	20.6
11	19	47.4	47.4	5.3
12	2	100	0	0
Genotypes (GGC)n				
7-7	1	0	100	0
7-10	7	28.6	14.3	57.1
7-11	1	100	0	0
9-10	0			
1-10	26	46.2	34.6	19.2
10-11	13	38.5	53.9	7.7
10-12	1	100	0	0
11-11	2	50	50	0
11-12	1	100	0	0

Variations in the expression level are analysed for each allele and genotype. OE, overexpression; NV, no-variation; UE, under-expression.

detected within the 52 cases of breast cancer were within the 27 cases of IDC and both overexpressed the *eRF3a/GSPT1* and eRF1 mRNAs (samples identified with # in Fig. 2A). Interestingly, both tumors were positive for RE and RP immunoexpression.

Translation termination activity of eRF3a forms encoded by the GGCn alleles. We used complementation experiments in eRF3a-depleted cells to analyze the translation termination activity of the 5 eRF3a variants encoded by the 5 known alleles of *eRF3a/GSPT1* gene. Parallel cultures of 559C cells, a cell line expressing a *lacZ* reporter gene interrupted by a premature stop codon, were depleted in eRF3a by electroporation of a plasmid expressing a siRNA, si-3a1, specifically targeting *eRF3a/GSPT1* mRNA. Three days after electroporation, cells were re-electroporated with the plasmids expressing the eRF3a variants. Three days later, the effect on stop codon readthrough was analyzed by measuring the β -galactosidase activity. Cells electroporated with the empty vector pSuper and re-electroporated with plasmid pBK-CMV served as a negative control (Fig. 3A, lane sup/pBK). Cells electroporated with plasmid expressing si-3a1 and re-electroporated with plasmid pBK-CMV served as standard for readthrough level promoted by eRF3a depletion (Fig. 3A, lane 3a1/pBK). For each electroporation experiment, the amount of endogenous eRF1 and eRF3a overexpressed proteins was verified by Western blot analysis. As shown

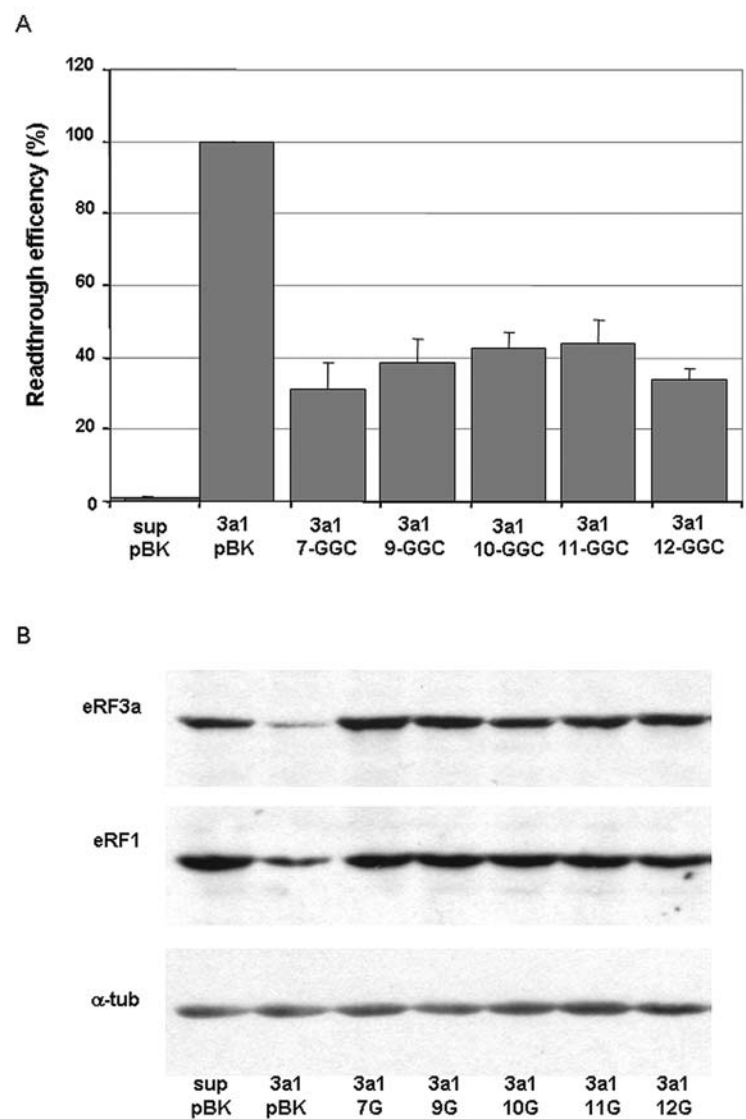


Figure 3. Translation termination activity of eRF3a forms encoded by the different GGC alleles. (A) Readthrough efficiencies of eRF3a proteins were calculated by dividing the β -galactosidase activity in each sample by the β -galactosidase activity in the control cell extract 3a1/pBK. The results of readthrough assays were normalized to the level of 3a1/pBK-CMV electroporated cells which served as standard for readthrough level promoted by eRF3a depletion (lane 3a1/pBK). The results were expressed as the mean of five experiments; the error bars show the standard errors of the mean. (B) Western blot analysis performed 3 days after the second electroporation of cells expressing eRF3a variants encoded by the different GGC alleles (7G, 9G, 10G, 11G, and 12G) using anti-eRF3a and anti-eRF1 antibodies. Equal protein loading was verified by the detection of α -tubulin (α -tub).

in Fig. 3B, all of the eRF3a variants were expressed at the same level. Our results show no significant differences in the readthrough efficiencies whatever the eRF3a variant overexpressed (Fig. 3B). This suggests that the length of the glycine stretch encoded by the GGC repeat has no detectable effect on eRF3a activity in translation termination.

Discussion

eRF3 is a critical protein, playing a role in several points of cell regulation. The N-terminal domain of eRF3a has been shown to participate in essential protein interactions necessary for different eRF3a functions, namely the formation of the translation termination complex (10), coupling termination with re-initiation in successive cycles of translation (23) and as an initiator of NMD (24). Apart from its canonical roles in translation, the N-terminal domain of eRF3a was also reported to interact with various proteins with different

biological functions (9) and also to act as a regulator of apoptosis, being proteolytically processed into an isoform that contains a conserved N-terminal IAP-binding motif (25). As it is implicated in essential cellular processes, modifications in the amino-terminal domain of eRF3a might be of crucial relevance.

We have previously showed that the longer allele (12-GGC) of the GGCn polymorphism in eRF3a/GSPT1 exon 1 is present in 8% of gastric cancer patients (11/139), and thus is associated with ~20-fold increased risk of gastric cancer (15). We also demonstrated that *eRF3a/GSPT1* mRNA expression is up-regulated in 70% of the intestinal type gastric tumors analysed (6). However, the correlation between the patient genotype and the levels of *eRF3a/GSPT1* expression was not established.

Herein, we report the presence of the 12-GGC allele in 5% (7/137) of the breast cancer patients, in contrast with zero in the control population (0/135). Given the rarity of

the allele, the statistical results need to be strengthened by replication in other populations. When both gastric (15) and breast cancer populations are grouped together and compared with the control population, the 12-GGC allele is present in 6.52% of the cancer cases, with an allele frequency of 3.8%, corresponding to a 15-fold increased risk for cancer (OR=15.49; 95% CI=0.93-257.43). Therefore, we propose that the presence of the 12-GGC allele, by itself, may provide a new useful marker for cancer susceptibility.

Relative quantification of mRNA in breast tumor tissues revealed an overexpression of *eRF3a/GSPT1* in 44% of the tumors analysed. It is known that cell growth and proliferation rates depend critically on the rate of protein synthesis. To investigate if variations in *eRF3a/GSPT1* gene expression pattern are quantity adjustments dependent on the cell translation rates, we quantified the levels of expression of the other translation termination factors. Because eRF1 is the major factor in translation termination, we analysed the pattern of eRF1 gene expression assuming that it is dependent on cell translation rates. No variations between normal and tumor tissues were detected in 44% of the patients analysed. Moreover, there are no correlations between eRF1 and *eRF3a/GSPT1* transcript levels. In what concerns eRF3b, an isoform of eRF3a, no transcripts were detected in the majority of the patient tissues analysed and no correlation between *eRF3a/GSPT1* and *eRF3b/GSPT2* expression was detected. Therefore, there is no parallel between *eRF3a/GSPT1* transcript levels and additional cell requirements for increased translation.

Our results provide the first indication suggesting that the length of the GGC repeat is associated with the variations of *eRF3a/GSPT1* transcript levels in tumor samples. The longer allele (12-GGC) was only found in patients with *eRF3a/GSPT1* overexpression in tumor samples. In contrast, the shorter allele (7-GGC) was mostly associated to patients with *eRF3a/GSPT1* underexpression.

A number of hypotheses can be raised to explain the association of the 12-GGC allele with cancer development. It can involve a dominant gain of function effect by shifting the protein's conformation, thus altering its function or achieving toxicity. Loss of function can also occur, by interfering with DNA methylation, RNA processing or translation efficiency. As a first approach, we looked for differences in the efficiency of eRF3a with different lengths of polyglycine stretches in the N-terminal domain. However, the GGC repeat length showed no effect in eRF3a activity, in what concerns its role as a translation termination factor.

It is known that protein synthesis is coupled with cell cycle progression and is regulated in response to nutrient availability and mitogenic stimulation. However, the transduction pathways activated do not stimulate the translation of all the mRNAs equally (3,26). The expression of the components of the translation machinery might be selectively regulated, increasing the translation rate of specific oncogenic transcripts (26,27). Our recent results revealed that eRF3a depletion inhibited translation and cell cycle progression in HCT116 cell line via mTOR pathway inhibition (28). After activation by mTOR, S6K phosphorylates the ribosomal protein S6, leading to an increase in translation of a subset

of mRNAs (29). Therefore, it is tempting to speculate that eRF3a overexpression detected in different kinds of tumors might be responsible for mTOR activation, leading to differential expression of specific targets and consequent malignant transformation. It is noteworthy that the 12-GGC allele was only detected in patients who are simultaneously RE and RP positive, which are important markers usually taken in consideration for prognostic evaluation and therapeutic decisions (22).

Understanding *eRF3a/GSPT1* gene regulation and its relation with cell cycle progression and cellular proliferation may have prognostic value and potential therapeutic applications. A better knowledge of the influence of the polyglycine stretch on the role of eRF3a will allow to establish whether eRF3a could be used as a new biomarker for breast and other types of hereditary cancer.

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